**Use of modulators of the signal transduction pathway via
the protein kinase Fyn in the treatment of tumour diseases**

The present invention describes the use of modulators of
5   the signal transduction pathway via the protein kinase Fyn
(e.g. Rho-kinase inhibitors) in the treatment of tumour
diseases, especially in the therapy of neuroblastomas.

Neuroblastomas are malignant cancer conditions of the
10   peripheral sympathetic nervous system which occur during
childhood. They are one of the most frequent malignant
cancer conditions of childhood. Each year about 150-200
children in Germany are afflicted by this tumour, which in
advanced stages is usually incurable. The course of the
15   disease is different in individual cases and ranges from
spontaneous regression to a progressive course and the
formation of metastases. The tumour develops from
precursor cells of the autonomous nervous system, which
controls involuntary functions such as the cardiovascular
20   system and bowel and bladder function. In total, about
40 % of children afflicted die within the first five
years.

A genetic criterion used as the basis for a poor prognosis
25   is amplification of the MYCN gene, which results in
deregulated expression of the N-Myc protein in the tumour
tissue. N-myc is a transcription factor which can both
positively and negatively control expression of the gene.
Using a cell culture model it has been shown that Myc
30   proteins regulate both cell growth and cell proliferation.

Current clinical investigations make use of three criteria
in establishing a prognosis for neuroblastoma:

the tumour stage, the age of the patient, and amplification of the MYCN gene. Amplification of the MYCN gene, is, however, not a reliable criterion because tumours which do not exhibit the amplified MYCN gene can

5 also develop.

In that context, a problem of the present invention is to utilise genetic differences in those signal transduction pathways which control proliferation and differentiation

10 of the neuroblastoma for establishing a prognosis and for therapy in tumour diseases, especially neuroblastoma.

A problem of the present invention is to make available new methods of treating tumour diseases, especially

15 neuroblastoma.

By means of a microarray analysis, the expression of a large number of genes in an arbitrary number of samples from many patients, all of whom are suffering from

20 neuroblastoma, is measured in parallel. A statistical analysis is then carried out, in which clinical parameters are correlated with the expression data and consequently genes are identified which are causally involved in tumour progression. By identifying genes which are causally

25 involved in tumour progression we open up the possibility of causal therapy for the disease.

Expression of the identified genes correlates with specific tumour stages. Surprisingly, a large proportion

30 of those genes belong to a signal transduction pathway which proceeds via the protein kinase Fyn. This signal transduction pathway is known to regulate cell adhesion, cell proliferation and differentiation in cell cultures.

A change in cell adhesion is responsible for the formation
of metastases; cell differentiation and proliferation
control tumour growth itself. It is shown that the data
can be validated by independent measurement methods. By
means of Western blot methods it has been shown that there
is a connection between the activity and/or expression of
Fyn-kinase and the stage of the tumour. All the
measurements show that signal transduction by Fyn is
switched off in advanced tumour stages. The expression
analyses underline the role of the Fyn signal transduction
pathway in the formation of metastases.

In addition we demonstrate that Fyn has a causal role in
the mentioned processes in neuroblastoma. Using two
different neuroblastoma cell lines it is shown that the
expression of Fyn, that is to say the reactivation of
signal transduction, results in growth arrest, in
increased adhesion and in the differentiation of
neuroblastoma cells in culture. We have consequently
demonstrated that the loss of signal transduction by Fyn
is causal in the mentioned biological processes.

By means of a microarray analysis of human tumour samples
of neuroblastoma it has been found, surprisingly, that the
signal transduction pathway via the protein kinase Fyn
regulates tumour growth and the formation of metastases.
Furthermore, it was found that signal transduction by Fyn
is switched off in the advanced tumour stage. Influencing
the signal transduction pathway via Fyn-kinase, especially
(re-)activation of the signal transduction pathway via
Fyn, which is down-regulated in the neuroblastoma,
constitutes a possibility for therapy in the treatment of
neuroblastomas.

By using modulators of the signal transduction pathway via the protein kinase Fyn it is possible, in accordance with the invention, to prevent tumour growth and the formation of metastases and consequently to treat tumour diseases (especially neuroblastoma).

A further embodiment of the invention relates to modulators of the signal transduction pathway via the protein kinase Fyn, the modulators being inhibitors of enzymes which are inhibited directly or indirectly by active Fyn.

A further embodiment of the invention relates to modulators which result in an increase in Fyn activity in the tumour cells.

A further embodiment of the invention relates to modulators, the modulators being inhibitors of the protein kinase CSK, inhibitors of Rho-kinase, inhibitors of MAP-phosphatase or activators of protein kinase C.

A further embodiment of the invention relates to modulators which inhibit the proteolytic degradation of Fyn-kinase *in vivo*.

A further embodiment of the invention relates to modulators, the modulators being inhibitors of phosphatases that antagonise Fyn.

A further embodiment of the invention relates to modulators which contribute to an increase in signal transduction by Fyn.

In addition, the invention relates to the use of
modulators of the signal transduction pathway via the
protein kinase Fyn in the production of a medicament for
5   the suppression of the formation of metastases of tumours
in the early stage, it being possible for the tumour to be
a paediatric or adult tumour.

The invention relates furthermore to the use of modulators
10   as described above as a constituent of a pharmaceutical
composition, characterised in that the composition, in
addition to comprising the modulator as active ingredient,
optionally comprises carrier substances and/or adjuvants.

15   A further embodiment of the invention relates to
modulators as described above, characterised in that the
modulator is present in the form of a pharmacologically
acceptable salt, solvate, hydrate or a pharmacologically
acceptable formulation.
20

A further embodiment of the invention relates to
modulators as described above, characterised in that the
modulator is present in the form of a prodrug comprising
the modulator and at least one pharmacologically
25   acceptable protecting group which is removed under
physiological conditions.

A further embodiment of the invention relates to
modulators as described above, characterised in that the
30   modulator is administered orally, parenterally, rectally,
by inhalation, transdermally or intranasally.

Detailed description of the invention

A corresponding scientific study on this subject has been
published by B. Berwanger, O. Hartmann, E. Bergmann, S.
Bernard, D. Nielsen, M. Krause, A. Kartal, D. Flynn, R.
5   Wiedemeyer, M. Schwab, H. Schäfer, H. Christiansen and M.
Eilers: "Loss of a FYN-regulated differentiation and
growth arrest pathway in advanced stage neuroblastoma" in
Cancer Cell, Vol. 2, November 2002, page 377-386, to the
full content of which reference is made.
10

In order to gain an insight into the molecular sequence of
the development of neuroblastomas, expression profiles of
94 individual tumour tissue samples were produced, using a
human Unigene 4608 cDNA chip. Each chip is hybridised
15   with cDNA as reference which is derived from a human
neuroblastoma cell line (SHEP). The tumours were so
selected that they reflected the distribution of the
tumour stages and the MYCN amplification of the tumour
bank as a whole. In order to allow comparisons between
20   the individual spots and the arrays, each signal was
corrected in relation to its background, and the $\log_2$-
transformed on/off regulation intensity ratios were
calculated and standardised. A two-sample t statistic
with adjusted p values was used in order to identify
25   differently expressed genes (Callow et al., 2000). The
adjusted p values simultaneously correct the testing of
4608 genes and estimate the overall probability of
detecting an incorrect gene (see methods).

30   We found 123 differently expressed genes when we compared
MYCN-amplified (n = 17) and non-amplified (n = 77) tumours
(adjusted p value < 0.05). These genes were used in a
non-controlled, hierarchical cluster, and the analysis

showed that all tumours except three were correctly
assigned to one of the two classes. Functional annotation
showed that most of the genes that were switched on in
MYCN-amplified tumours encode proteins which play a role

5   in protein synthesis, in metabolism and in regulation of
the cell cycle; this agrees with the microarray data
produced by using inducible systems in cell culture.
These results show that Myc proteins regulate both cell
growth and also cell proliferation and that the models

10   developed using the cell culture extend to the formation
of the neuroblastoma. The expression of a series of genes
which were identified as target genes of (c-)Myc in cell
culture experiments was significantly different between
the two classes of tumours. A large group of genes most

15   of which were switched off in MYCN-amplified tumours
encode proteins which are involved in multi-stage signal
transduction pathways. This shows that Myc proteins
constitute a negative feedback signal for signal
transduction pathways. Surprisingly, the cell cycle genes

20   which increasingly appear in MYCN-amplified tumours encode
proteins which are known to function as a checkpoint
response or in the G2 or M phase of the cycle, which
points to a new function of Myc in checkpoint control and
late in the cell cycle.

25

The deregulation of those genes can, in simple manner,
reflect the advanced tumour stage of most MYCN-amplified
tumours (Table 1). In order to exclude that possibility,
we compared their expression between stage 4 of MYCN-

30   amplified and non-amplified tumours. The expression of
all genes analysed by us was regulated by MYCN
amplification, irrespective of the tumour stage.
Conversely, the deregulation could reflect direct

regulation by N-Myc. This interpretation is in agreement with the fact that many E-boxes are present in the promoter and introns of the MAD2, CENPE and AURORA2 genes (Figure 1, Field e). Chromatin immunoprecipitation did indeed show that N-Myc is bound *in vivo* to the E-boxes of MAD2 genes. From that we concluded that at least some of the genes identified by us are direct target genes of N-Myc.

Table 1

		STAGE					Total
		1	2	3	4	4S	
MYCN	Non-amplified	19	8	17	21	12	77
	Amplified	1	1	4	8	3	17
Age	< 12 months	18	1	11	5	15	50
	> 12 months	2	8	10	24	0	44
Age	Mean (months)	4.3	23.4	28.7	42.6	4.0	23.3
	Standard deviation (months)	4.2	17.8	41.1	33.6	3.4	31.6
Total		20	9	21	29	15	94

Table 1: Clinical parameters of 94 patients taking part in this study. Age and stage are highly disparate, and because of the low number they could not be analysed independently.

An initial study showed that, among the non-MYCN-amplified tumours, stages 1 and 4, but not stages 2, 3 and 4S, show differences in their expression profiles (data not shown). We found 36 indicative genes which were differently expressed in tumours in stage 1 (n = 19) and in stage 4 (n = 21) (adjusted p value < 0.2). That set of genes showed a slight overlap with the group of genes which differentiates MYCN-amplified tumours from non-amplified tumours; functional annotation of the genes showed that the genes which encode proteins which play a role in

metabolism and protein synthesis were apparently absent
from the group of stage-specific genes, in contrast to the
genes which were characteristic of MYCN-amplified tumours.
In contrast, a characteristic percentage of genes which
5   were differently expressed encodes genes which are
involved in signal transfer by the non-receptor tyrosine
kinase Fyn and the actin cytoskeleton; these genes were
down-regulated in co-ordinated manner in the advanced
stage of the neuroblastoma. This group includes Fyn
10   itself, actin filament binding protein (AFAP), a protein
which binds to Src and Fyn-kinase and activates the
latter; α-catenin (CTNNA1), an actin-binding protein whose
binding to β- and γ-catenin is regulated by Fyn-dependent
phosphorylation; the neural cell adhesion protein NRCAM,
15   which signals by way of non-receptor tyrosine kinases and
the actin-binding proteins tropomodulin and MARCKS.

Western blot confirmed the reduced expression of Fyn in
tumours of stage 4 compared to stage 1; in addition, the
20   experiment showed, in agreement therewith, slower
migration of the Fyn protein in extracts from all tumours
of stage 1 compared to those of stage 4, which shows that
the autophosphorylated (active) form is present.
Phosphatase treatment confirmed that the differing
25   migration came about as a result of the phosphorylation.
In conformity with those observations, a high Fyn-kinase
activity was obtained in tissue samples from tumours of
stage 1, for which immunocomplex kinase assays were used
which measure both autophosphorylation and also
30   phosphorylation of the exogenous substrate, enolase (Wolf
et al., 2001). In contrast, the Fyn-kinase activity in
extracts from tumours of stage 4 was variable and, on
average, substantially lower.

In order to test whether Fyn plays a role in
differentiation and regulation of cell proliferation of
neuroblastoma cells, we used a transient transfection in
5   order to express Fyn in SH-SY5Y cells - a human
neuroblastoma cell line derived from a stage 4 tumour
(Pahlman et al., 1981). Expression of wild-type Fyn
induced the extension of multiple neurites and overt
morphological characteristics of differentiation.
10   Staining with antibodies directed against cyclin A as a
marker protein of cell proliferation showed that the cells
which expressed active Fyn had exited from the cell cycle.
In contrast, cells which expressed a kinase-negative
allele (FynK299M) showed no signs of morphological
15   differentiation. In conformity with the role of AFAP in
the activation of Fyn, the expression of a constitutively
active allele of AFAP induced morphological changes highly
reminiscent of active Fyn, whereas a dominant negative
allele of AFAP does not influence differentiation.

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We repeated the experiments in IMR-32 cells, a human
neuroblastoma cell line that carries an amplified MYCN
gene (Clementi et al., 1986). Similarly to the results
obtained in SH-SY5Y cells, the expression of active Fyn-
25   kinase induced neurite extension and cell cycle exit.
This shows that the induction of differentiation by Fyn
occurs even in the presence of an amplified MYCN gene.
This agrees with the finding that the expression of FYN,
AFAP, NRCAM and CTNNA1 is down-regulated equally in MYCN-
30   amplified tumours and in non-amplified tumours, based on
tumours in stage 1.

In all, we identified two genetic programs which regulate
the development of neuroblastomas, one which is controlled
by amplification of the MYCN gene and a second, stage-
specific program of gene expression. The two programs
5   are, to a high degree, independent of one another because
(a) the two groups of genes show little overlap, (b) the
genes are down-regulated by MYCN amplification
irrespective of tumour stage and (c) the tumour-stage-
specific genes are down-regulated irrespective of MYCN
10   amplification. The deregulated expression of MYCN
activates genes encoding proteins which are involved both
in advancement of the cell cycle and also in cell growth
and suppresses genes encoding proteins involved in multi-
stage signal processes in a human tumour. These findings
15   show, specifically, that Myc proteins control gene
expression in the G2 phase of the cell cycle and activate
genes which are involved in checkpoint processes.

Our data show that Fyn-kinase regulates the proliferation
20   and differentiation of neuroblastoma cells *in vivo*; this
is likewise supported by the finding that the stage-
specific expression profile is predictive of survival.
Detailed expression profiles of individual tumour stages
showed that the down-regulation of Fyn is most marked
25   between stages 1 and 2, which correlates with the
formation of metastases in the local lymph nodes. The
down-regulation of Fyn and also the modified cell adhesion
control the formation of local metastases *in vivo*.

30   Active Fyn can perform its function in a number of ways:
In neuronal cells, non-receptor tyrosine kinases
phosphorylate Rho-GAP, which results in inactivation of
Rho and induction of differentiation. It was found that

there are molecules in signal transduction pathways which
are inhibited by Fyn signalling, which makes them into
candidates for a therapeutic intervention. In accordance
with the invention, influencing the signal transduction
5   pathway downstream of Fyn provides a therapeutic approach
for advanced-stage neuroblastomas.

Preferred target molecules in the Fyn signal transduction
pathway are those which are inhibited by Fyn signal
10   transduction.

Preferred modulators are inhibitors of enzymes which are
directly or indirectly inhibited by active Fyn.

15   Further preference is given to modulators which result in
an increase in Fyn activity in tumour cells (especially in
neuroblastomas).

Special preference is given to inhibitors of the protein
20   kinase CSK, which is expressed in neuroblastomas and is a
negative regulator of Fyn *in vivo*.

Preference is moreover given to modulators which inhibit
the proteolytic degradation of Fyn-kinase *in vivo*, e.g.
25   general inhibitors of the proteasome (LLNL, MG132) or
specific inhibitors of the E3 ligases involved.

Further preferred modulators are inhibitors of
phosphatases which antagonise Fyn, e.g. MAP-kinase
30   phosphatase 1.

Preference is also given to modulators which contribute to
an increase in signal transduction by Fyn, e.g. Rho-kinase

inhibitors, MAP-phosphatase inhibitors or activators of protein kinase C.

The present invention furthermore encompasses pharmacologically acceptable salts, solvates, hydrates or pharmacologically acceptable formulations of the described modulators.

Examples of pharmacologically acceptable salts are salts of physiologically acceptable mineral acids such as hydrochloric acid, sulphuric acid and phosphoric acid or salts of organic acids such as methanesulphonic acid, p-toluenesulphonic acid, lactic acid, acetic acid, trifluoroacetic acid, citric acid, succinic acid, fumaric acid, maleic acid and salicylic acid. The modulators according to the invention may be solvated, especially hydrated. The hydration may occur, for example, during the preparation process or as a consequence of the hygroscopic nature of the initially anhydrous compounds. When the described modulators contain asymmetric C atoms, they may be present either in the form of mixtures of diastereomers, mixtures of enantiomers or in the form of optically pure compounds.

The pharmaceutical compositions according to the present invention comprise at least one of the described modulators as active ingredient and, optionally, carrier substances and/or adjuvants.

The prodrugs, to which the present invention also relates, consist of a modulator according to the invention and at least one pharmacologically acceptable protecting group which is removed under physiological conditions, for

example an alkoxy, aralkyloxy, acyl or acyloxy group, e.g. an ethoxy, benzyloxy, acetyl or acetoxy group.

The present invention relates also to the use of the modulators in the production of medicaments for the prevention and/or treatment of tumour diseases (especially neuroblastoma). In general, the modulators are administered either individually or in combination with any other desired therapeutic agent, using the known and acceptable methods. Such therapeutically useful agents can be administered by one of the following routes: orally, for example in the form of dragées, coated tablets, pills, semi-solid substances, soft or hard capsules, solutions, emulsions or suspensions; parenterally, for example in the form of an injectable solution; rectally in the form of suppositories; by inhalation, for example in the form of a powder formulation or spray, transdermally or intranasally. For the production of such tablets, pills, semi-solid substances, coated tablets, dragées and hard gelatin capsules, the therapeutically usable product can be mixed with pharmacologically inert, inorganic or organic pharmaceutical carrier substances, for example with lactose, sucrose, glucose, gelatin, malt, silica gel, starch or derivatives thereof, talcum, stearic acid or salts thereof, skimmed milk powder and the like. For the production of soft capsules, pharmaceutical carrier substances such as, for example, vegetable oils, petroleum, animal or synthetic oils, waxes, fats and polyols can be used. For the production of liquid solutions and syrups, pharmaceutical carrier substances such as, for example, water, alcohols, aqueous saline solution, aqueous dextrose, polyols, glycerol, vegetable

oils, petroleum and animal or synthetic oils can be used. For suppositories, pharmaceutical carrier substances such as, for example, vegetable oils, petroleum, animal or synthetic oils, wax, fat and polyols can be used. For aerosol formulations, compressed gases that are suitable for the purpose can be used, such as, for example, oxygen, nitrogen and carbon dioxide. The pharmaceutically acceptable agents may also comprise additives for preserving and stabilising, emulsifiers, sweeteners, flavourings, salts for altering the osmotic pressure, buffers, encapsulation additives and anti-oxidants.

Combinations with other therapeutic agents can include other active ingredients which are customarily used in the treatment of tumour diseases (especially neuroblastoma).

For the prevention and/or treatment of the diseases described above, the dose of the modulators according to the invention can vary within wide limits and can be adjusted to individual requirements. In general, a dose of from 0.1 µg to 100 mg/kg of body weight per day is suitable, a preferred dose being from 0.5 to 10 mg/kg per day. In suitable cases, the dose may also be below or above the stated values.
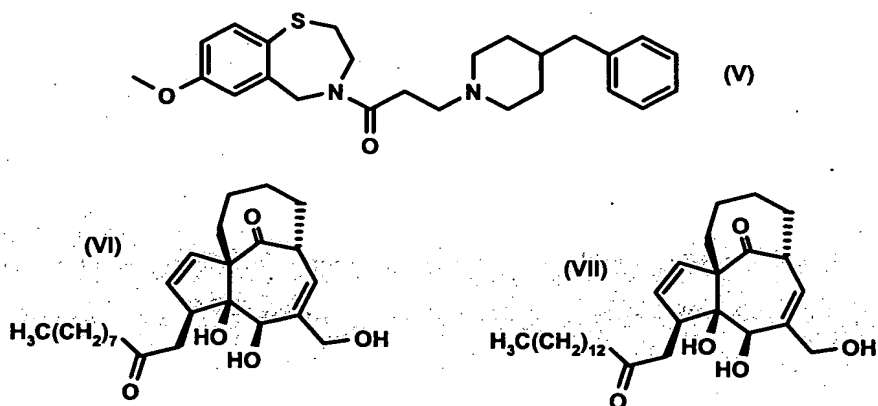
Examples

Examples of Rho-kinase inhibitors are described in EP0370498, US4997834, EP0956865, US6218410, US4678783, US6153608, EP0885888, WO0168607 and WO0156988. Specifically, there may be mentioned herein compounds **I** (fasudil), **II** (hydroxyfasudil), **III** and **IV**:

(I)

(II)

(III)

(IV)
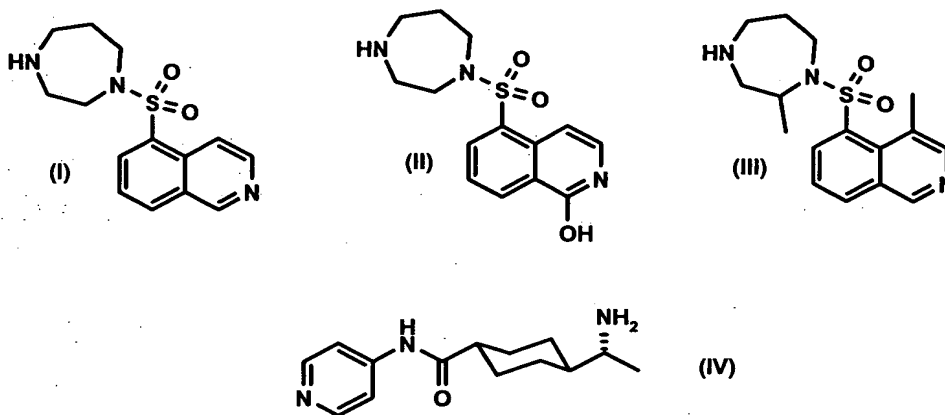
Examples of activators of protein kinase C are compound **V**, the compound EP-70905 of Europeptides, the natural substances bryostatin, teleocidin, aplysiatoxin and also esters of phorbol and ingenol. Further compounds such as **VI** and **VII** are described in J. D. Winkler et al. J. Am. Chem. Soc. 1999, 121, 296-300.

(V)

(VI)

(VII)

An example of an MAP-kinase phosphatase 1 inhibitor is the compound MX 7091 of Maxima Pharmaceuticals.

An example of a CSK inhibitor is the natural substance staurosporine.

5 Materials and methods

Microarray experiments

The chip used comprises the cDNA set gf200 of Research
10 Genetics (http://www.resgen.com) and, in addition, 100
cDNAs that had already been described previously as
potentially suitable for the prognosis of neuroblastoma
development (for details see http://www.imt.uni-
marburg.de). Each cDNA was spotted twice per chip. The
15 chips were produced as described in Hegde et al., 2000,
using a GMS 417 arrayer.

An initial histochemical study of a series of 100 randomly
selected tumours revealed that approximately 95 % of the
20 tumours contained less than 5 % non-tumour cells in the
tissue samples (see Bergmann et al., 2001). Therefore, no
further attempt was made to dissect the tumour tissue
before preparation of the RNA. Total RNA from the
neuroblastoma tissue and SHEP was isolated using a Qiagen
25 RNA isolation kit in accordance with the manufacturer's
instructions. 40 μg of total RNA were used in order to
produce Cy3 and Cy5 fluorescently labelled cDNA in
accordance with the published protocol
(http://brownlab.stanford.edu). The chips were scanned
30 using a GMS 418 fluorescence scanner, and the images were
analysed using IMAGENE 3.0 software. The expression data
were confirmed either by Northern blot analysis or by
real-time RT-PCR assays.

Standardisation and quality control

ImaGene 3.0: The software parameters such as "signal ranges" or "spot detection threshold" (for details see the ImaGene user manual) were optimised for maximum reproducibility prior to image analysis in our experiment. For each spot the median signal and the background intensities for both channels are obtained. In order to determine the differences between spots, the corrected background ratio of the two channels was calculated and $\log_2$-transformed.

In order to balance the fluorescence intensities of the two dyes and also to allow a comparison of the expression level across experiments, the raw data were standardised. First, we used a pin-wise intensity-dependent standardisation (Yang *et al.*, 2002) in order to correct for inherent bias on each chip (the lowess scatter-plot smoother). In a second step, a general standardisation was performed in order to centre the log-ratios for each array at 0 (in order to take into account general staining and scanner effects). Because each gene was spotted on the chip twice, the mean log-ratios M were calculated from the replicates. If the replicates differed by more than a factor of four or if the background intensity was higher than the signal intensity, that gene was excluded from the array.

Statistical analysis

The final data matrix consisted of 4608 standardised gene expression measurements ($\log_2$-ratios) from 94 individual tumours (with missing values). In order to compare the expression profile between two independent groups, a two-sample t statistic was used for each gene. In order to

account for multiple testing, we calculated the adjusted p
values for each gene, in the course of which we used a
step-down permutation algorithm (Westfall and Young, 1993,
algorithm 4.1). This strategy had been applied to
5   microarrays before (Callow et al., 2000). The permutation
algorithm provides a powerful check on the family-wise
error rate (FWER) and takes into account correlation of
the variables (genes). The procedure does not rely upon a
normality assumption; it is assumed that the t statistic
10   has asymptotically the same null distribution for all
genes (or that the p values are monotonic in the observed
t statistics across the genes).

Cluster analysis
15   Prior to cluster analysis, the expression profile of each
gene was centred by subtraction of the mean observed
value. Average linkage hierarchical clustering was then
performed for genes and also for chips, in the course of
which the Euclidean distance metric as implemented in the
20   program J-Express was used (Dysvik and Jonassen, 2001).

Western blots, immunoprecipitation, phosphatase treatment
The following antibodies were used in Western blots,
immunofluorescence experiments and immunoprecipitations:
25   α-Fyn: (sc-434); α-cdk2 (sc-163), α-cyclinA (sc-751), all
from Santa Cruz. Neuroblastoma tissue was lysed as
described (Bergmann et al., 2001).
For the phosphatase treatment, 500 μg of cellular proteins
were incubated overnight at 4°C with 5 μg of Fyn-antibody
30   bound to protein G beads. Immunocomplexes were incubated
either with λ-protein phosphatase (NEB) or with λ-protein
phosphatase and phosphatase inhibitors. Immunocomplexes
were electrophoretically separated by means of a 10 % SDS

PAGE, transferred to a PVDF membrane and detected with an α-Fyn antibody.

In vitro kinase assays

500 μg of cellular proteins were immunoprecipitated with 5 μg of α-Fyn antibody bound to protein G beads, washed and equilibrated in kinase assay buffer, incubated for 15 minutes with 10 μCi γ-ATP (Amersham) and 0.125 mg/ml of enolase, electrophoretically separated by means of a 10 % SDS PAGE and dried. The results were visualised on a Fuji Phosphorimager and quantified using Bildeich software.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as previously described (Bouchard et al., 2001). Nuclear extracts were immunoprecipitated overnight at 4°C with 3 μg of α-N-Myc antibody or control antibody bound to protein A and protein G beads. For PCR analysis, a specific primer pair for intron 1 of prothymosin alpha (as positive control) and also primer pairs amplifying the indicated regions of the MAD2 gene were used. Primer sequences are available upon request.

Cell culture experiments

SH-SY5Y and IMR-32 neuroblastoma cell lines were cultured in RPMI 1640 supplemented with 10 % heat-inactivated FCS. CMV-driven expression constructs encoding wild-type Fyn (Fynwt)and FynK299M have been described (Wolf et al., 2001). Plasmids encoding AFAPΔLZ and AFAPΔ180-226 have been described (Baisden et al., 2001). For transient transfections, the cells were first allowed to grow for 6 hours on cover slips coated with a 1:5 dilution of

Matrigel (Becton-Dickinson). Transfection was performed
by using 5 μg of DNA and also a Lipofectin reagent
(Invitrogen). The cells were fixed with paraformaldehyde
after 60 hours, washed and stained with a monoclonal α-Fyn

5   antibody (Santa Cruz) or an α-AFAP rabbit polyclonal
antibody (Baisden *et al.*, 2001).

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